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Increased soluble EGF after ischemia is accompanied by a decrease in membrane-associated precursors

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Schaudies, R. Paul, and John P. Johnson. Increased soluble EGF after ischemia is accompanied by a decrease in membrane-associated precursors. *Am. J. Physiol.* 264 (*Renal Fluid Electrolyte Physiol.* 33): F523-F531, 1993.—We have characterized the distribution of immunoreactive epidermal growth factor (irEGF) in control and ischemia-injured rat kidneys. Kidneys that had undergone ischemic injury contained levels of soluble irEGF that were six times those of uninjured kidneys. The predominant forms of soluble irEGF were native and des-Arg-epidermal growth factor (EGF), both of which are biologically active. Crude membrane fractions from whole kidneys were solubilized in Triton X-100 and tested for irEGF. Amounts of irEGF were slightly decreased in the ischemia-injured kidney membranes. However, when solubilized membrane fractions were digested with trypsin, which generates a single immunoreactive species which appears identical to native EGF, the amount of irEGF in control fractions increased 13-fold and the amount in injured fractions increased only 4-fold as measured by radioimmunoassay. To better characterize the membrane-associated irEGF, Triton X-100-solubilized membrane fractions from control animals were affinity purified and subjected to high-performance liquid molecular sieve chromatography. Three major peaks of material exhibited immunoreactivity to EGF antibodies, bound the EGF receptor, and stimulated [^{3}H]thymidine incorporation in growth-arrested fibroblasts. Trypsin digestion of the two high-molecular-mass peaks enhanced these activities. The third peak eluted with native EGF and showed no change in activity with trypsin addition. We propose that EGF is released from membrane-associated EGF precursors and can then act in an autocrine or paracrine fashion to promote cell growth after ischemia-induced acute renal failure.

immunoreactive epidermal growth factor; renal failure; epidermal growth factor precursors

EPIDERMAL GROWTH FACTOR (EGF) is a potent polypeptide mitogen that can be isolated from rodent submandibular glands (26, 31) and human urine (5, 10). EGF has been demonstrated to stimulate a variety of both short- and long-term biological responses in vitro (for recent reviews see Refs. 4, 7, 9). Despite the volume of information about EGF and its effects in vitro, its specific function in vivo remains undefined.

One of the possible sites for a biological function for EGF is the kidney. Both EGF and the messenger RNA for the EGF precursor are present in the kidney (15, 22). The EGF precursor mRNA levels in the mouse kidney are one-half of those in the male submandibular gland and five times the level in the female submandibular gland (22). EGF synthesized within the kidney is thought to be the primary source of urinary EGF. Urine from humans and rodents has been reported to contain both native (6 kDa) and high-molecular-mass forms (140 kDa) of immunoreactive (ir) EGF (17, 19). Recently, Breyer and Cohen (3) have reported the isolation of a high-molecular-mass EGF precursor from murine kidney membranes. This EGF precursor binds the EGF receptor and stimulates cell growth in a dose-dependent manner. Biological activity of the human EGF precursor was demonstrated by its ability to support growth of a mouse keratinocyte cell line that is dependent on EGF for growth (20). The presence of a biologically active EGF precursor in kidney membranes supports a role for EGF in renal function.

Several features of the recovery phase of experimental acute renal failure suggest that a mitogen such as EGF may be involved (24, 35). These features include increased transcriptional activity, DNA synthesis, mitosis, and cellular proliferation. The mechanism by which renal epithelial cells are stimulated to proliferate and differentiate remains unclear. EGF is a potent mitogen for renal epithelial cells (12) and is a possible mediator of cellular proliferation in the kidney. Recent evidence indicates that exogenously administered EGF enhances recovery of renal function in acute renal failure (12). In addition, the level of EGF receptors within the kidney increases after ischemic injury (23). These results indicate that renal EGF may be important in the recovery process.

Interestingly, both renal prepro-EGF mRNA and urinary excretion of EGF have been reported to decrease after ischemic induction of acute renal failure in the rat (23, 24). However, we have recently reported that the levels of irEGF (irEGF) in rat kidney increase fivefold after potassium dichromate-induced, or ischemia-induced acute renal failure (32). This apparent discrepancy between a decrease in EGF precursor mRNA while soluble levels of EGF are increasing raises the question of the origin of this renal EGF and supports the possibility that irEGF is stored in kidney membranes and released subsequent to injury.

This study was conducted to characterize the irEGF in the soluble and crude membrane fractions of kidney homogenates and to determine whether the EGF precursors are a probable source of the increase in renal EGF. Measurements were conducted in both control animals and those in acute renal failure induced by temporary ischemia to examine the role of endogenous EGF in acute renal failure. Triton X-100-solubilized crude membrane preparations from control animals indicated multiple high-molecular-mass EGF-containing proteins. EGF was released after trypsin digestion of the Triton X-100 extract and was present at 300 ng/g wet wt tissue, representing >90% of the total renal EGF. In the ischemia-injured kidneys the level of soluble, biologically active, EGF increased sixfold within 24 h postinjury. During the same time period the level of Triton-soluble trypsin-releasable EGF decreased to ~100 ng/g in the injured kidneys. This increase in soluble biologically

active EGF with an associated decrease in membrane-associated precursors supports the presence of an autocrine or paracrine healing mechanism in the kidney activated by injury whereby the precursor is cleaved to release the mature form of the growth factor.

MATERIALS AND METHODS

Epidermal growth factor. Rat EGF was purified and iodinated as described previously (31). A homologous radioimmunoassay for rat EGF was performed as described (31). Polyclonal antiserum P-1 was generated in a rabbit by two separate injections of 200 μg rat EGF in Freund's complete adjuvant followed by incomplete adjuvant. This preparation of antiserum has been characterized regarding its cross-reactivity with murine and human EGF (31). In the present experiments, samples (up to a 350-μl vol) or known amounts of irEGF were added to tubes containing 20,000 cpm ^{125}I-labeled irEGF (150–200 μCi/μg) with antiserum at a final dilution of 1:1,500,000. After incubations supernatants were aspirated and pellets were monitored for ^{125}I using a LKB-1442 Compugamma counter containing an internal radioimmunoassay (RIA)-calculating program. The assay has a sensitivity of 25 pg EGF.

Experimental protocol. Renal ischemic injury was generated by the method of Siegel et al. (33). Adult male Sprague-Dawley rats (250–325 g) were anesthetized with an intraperitoneal injection of pentobarbital sodium (65 mg/100 g body wt). Body temperature was maintained with a heat lamp and monitored via a rectal probe. A midline abdominal incision was made, and the aorta and renal arteries were carefully dissected from the surrounding tissue. Heparin (50 U/100 g) was administered via jugular vein catheter. After 10 min a vascular clamp was placed across the aorta proximal to the origin of the left renal artery and traction was put on a Silastic loop around the right renal artery distal to the origin from the aorta. Ischemia was maintained for 35 min. The 35-min renal clamp has been the most successful time point for generation of renal failure with a >90% survival rate. Control animals were treated in an identical manner without occluding the artery. At 24 h postreperfusion the animals were anesthetized as before and the kidneys were flushed with ice-cold saline, aseptically removed, and snap frozen in liquid nitrogen. In our ischemic model the peak in serum creatinine levels occurs at ~24 h and we therefore used this time point to measure EGF levels. This peak in serum creatinine indicates decreased renal filtration of the blood. This is also within the window of peak EGF levels (unpublished observations). Blood samples were taken just before ischemic insult and at time the animals were killed for measurement of serum creatinine with a Beckman Creatinine Analyzer 2. In rats experiencing 35-min bilateral renal artery clamp, serum creatinine levels rose from control values of 0.15 ± 0.05 to 2.3 ± 0.7 mg/dl at 24 h postischemia (n = 15). Whole frozen kidneys from each separate rat were homogenized by the method of Breyer and Cohen (3) in 10 vol 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.5) and 2.0 mM $MgCl_2$ using a Brinkmann Polytron (P-10 probe, speed setting 6, 45 s). Homogenates were centrifuged at 100,000 g for 30 min at 4°C. Supernatants were retained and designated the soluble fraction. The pellets were washed with the original volume of buffer and recentrifuged as above. This pellet represented the crude membrane fraction. The washed pellet was homogenized in 7 vol 20 mM HEPES (pH 7.5), 0.15 M NaCl, and 1% Triton X-100 and centrifuged as above. The supernatant containing solubilized membrane-associated proteins was retained for further characterization of irEGF species. Data presented for each group are averages of 15 animals.

Affinity chromatography. Anti-rat EGF antiserum R-2 (a separate preparation of antiserum from the one used for RIA measurements) was coupled to Affi Gel 10 (Bio Rad) according to the product insert as described (27). The resulting affinity resin had a rat EGF binding capacity of 5 μg/50 μl of a 50% resin slurry. Triton-solubilized extracts were incubated with anti rat EGF affinity resin (100 μl slurry/10 ml extract) at 4°C overnight on a rotator. This represents a 500-fold excess of EGF binding capacity than the amount of EGF equivalents measured in the nontrypsin-digested Triton X-100 extracts. This high excess is necessary to remove all of the trypsin-releasable EGF activity from the homogenate. Resin was pelleted by centrifugation (800 g) for 5 min at 4°C. More than 95% of the immunoreactivity was removed from the homogenate. The resin was washed five times with 1 ml 20 mM HEPES (pH 7.5; all chemical reagents were of analytical grade and obtained from Sigma Chemical, St. Louis, MO). Ammonium acetate (100 μl 0.1 M) containing 1% sodium dodecyl sulfate (SDS) was added to the washed resin (50 μl packed gel) and heated to 95°C for 5 min. An additional 900 μl of 0.1 M ammonium acetate were added, the tube was vortexed, and the resin was pelleted as above. This procedure was required because of the insolubility of the EGF-containing proteins at low pH. Heating at neutral pH in the presence of SDS was the most effective method for recovery of activity from the resin to maintain the total levels of Triton-extracted immunoreactivity both before and after trypsin digestion. The supernatant was removed and lyophilized. The dry residue was resuspended in either high-performance liquid chromatographic (HPLC) mobile phase (0.1 M ammonium acetate, pH 7.5) or polyacrylamide gel running buffer [tris(hydroxymethyl)aminomethane-glycine, pH 9.1].

Polyacrylamide gel electrophoresis. Nondenaturing polyacrylamide gel electrophoresis was conducted by the method of Davis (6) with slight modifications as described (31). This nondenaturing gel system is capable of separating native EGF from EGF lacking the COOH-terminal arginine residue (des-Arg-EGF). Preparation of des-Arg standard irEGF was conducted by incubation of submandibular gland EGF with carboxypeptidase B as described previously (31). After electrophoresis, lanes were separated and cut into 3.0-mm slices followed by overnight extraction in 1.0 ml RIA buffer (0.05 M sodium phosphate, pH 7.5, 0.1% wt/vol bovine serum albumin). Each extracted gel slice was assayed in duplicate by RIA using 350 μl of extract. Standard irEGF (2 μg) was run in a separate lane on the gel. Addition of 1% SDS to the standard did not affect the migration rate (data not shown). For isolation of the irEGF, 20% of the sample was run in one lane and 80% in a separate lane on the same gel. The migration rates were determined by conducting a RIA on the extracted gel slices from the lane containing 20% of the activity. The corresponding slices in the lane with 80% of the activity were pooled, and the immunoreactive material was eluted with a Hoeffer Electroeluter. Four slices near the top of the gel in the same lane were electroeluted as negative controls.

Enzymatic digestion. For soluble or Triton-extracted crude membrane preparations, 1 ml of Triton-extracted membranes was incubated with 100 μg trypsin (Sigma catalog no. T 8642, 12,000 U/mg) in 0.1 M sodium phosphate (pH 7.5) for 1 h at 37°C. The reaction was terminated by the addition of 500 μg soybean trypsin inhibitor (Sigma catalog no. T 9128, 1 mg inhibits 10,000 U trypsin) in 100 μl phosphate buffer. For the digestion of the HPLC-fractionated material, 100 μl were removed from each 250-μl sample (30-s fractions, 0.5 ml/min flow rate) and incubated with 25 μg trypsin for 1 h at 37°C followed by 125 μg soybean trypsin inhibitor as above. The samples were then assayed for immunoreactivity by RIA.

HPLC. Affinity-purified material from the Triton-solubilized crude membranes was chromatographed on a Bio-Sil TSK 250 column (300 × 7.5 mm) with a 100-μl sample volume and a flow rate of 0.5 ml/min of 0.1 M ammonium acetate (pH 7.5). One-half minute fractions were collected, and aliquots were assayed

by RIA as described above. Subsequent to fractionation by HPLC the eluates were assayed directly by RIA and also assayed by RIA after the eluate was digested with trypsin to release EGF from the high-molecular-mass precursors.

Radioreceptor assay. Binding to the EGF receptor on cultured human fibroblasts was conducted as described (31). Confluent monolayers of cells in 24-multiwell plates were incubated with radiolabeled ^{125}I-rat EGF (0.5 ng/ml, 400,000 cpm/ng) and increasing concentrations standard rat EGF or unknown in a total volume of 200 μl. Cultures were incubated at 37°C for 40 min. Medium was aspirated followed by four washes with ice-cold phosphate-buffered saline (PBS). Cells were solubilized in 1.0 ml 10% NaOH for 30 min at room temperature. Cell suspensions were transferred to centrifuge tubes, and ^{125}I was monitored using a LKB 1282 Compugamma counter.

[^{3}H]thymidine incorporation. Incorporation of [^{3}H]thymidine into growth-arrested human foreskin fibroblasts, cell line JPS1, was conducted in 24-multiwell dishes as described (29). Cells (4×10^4) were plated in each well in 0.5 ml minimal essential medium (MEM) containing 10% fetal bovine serum (FBS) and allowed to adhere overnight. The medium was aspirated, replaced with 0.5 ml MEM containing 2.0% FBS, and incubated for 48 h to growth arrest the subconfluent cultures. Additions of either irEGF or the affinity-isolated and native gel-separated extracts were added to the cells, incubated at 37°C for 20 h followed by the addition of 200 μl MEM, 2% FBS containing 2.0 μCi [^{3}H]thymidine, and then incubated for an additional 4 h at 37°C. The medium was aspirated, the cells were washed three times with cold MEM, and the DNA was precipitated in the wells by the addition of 0.5 ml ice-cold 5% trichloroacetic acid (TCA; wt/vol) and incubated on ice for 30 min. The TCA was aspirated, and the DNA was solubilized in 200 μl 1.0 N NaOH for 30 min at 37°C. The suspension was transferred to scintillation vials, and each well was rinsed with 225 μl 1.0 N HCl, which was added to the vial. Vials were monitored for ^{3}H using a LKB 1219 Rackbeta counter.

RESULTS

Quantitation of soluble irEGF. Levels of irEGF within the soluble and crude membrane preparations of control or postischemic rat kidneys, as determined by a homologous RIA, are presented in Fig. 1. HEPES-soluble irEGF levels in control kidneys are 14.25 ± 1.8, which is in agreement with an earlier report (25). Extraction of postischemic kidneys under identical conditions gave strikingly different results. The cytosolic fraction contained 93.9 ± 22 ng/g wet wt, representing a sixfold increase over control values. These values were unaffected by digestion of the soluble fraction with trypsin (data not shown). It should be noted that although this irEGF is soluble its exact location in the intact kidney has not been determined.

Characterization of soluble EGF. The irEGF in the soluble or cytosolic fraction was purified by affinity chromatography for further biochemical characterization. Native polyacrylamide gel electrophoresis was used to characterize the immunoreactive species because of its ability to separate forms of EGF that differ by as little as one charged amino acid (30). However, this system does not provide reliable information regarding the relative molecular masses of the different forms of EGF. The migration profile of the affinity purified material on nondenaturing native polyacrylamide gels is shown in Fig. 2*A*. Cytosol of control kidneys contains predominantly one immunoreactive form that comigrates with native

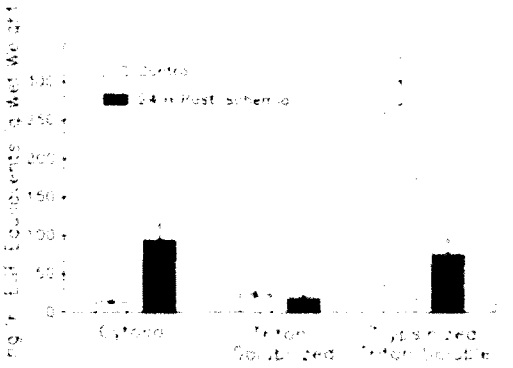

Fig. 1. Renal levels of rat immunoreactive epidermal growth factor (irEGF). Whole kidneys were homogenized and centrifuged at 100,000 *g* for 30 min at 4°C. High-speed supernatant is considered cytosolic supernatant. Resulting pellet was washed once and then homogenized in presence of 1% Triton X-100 and centrifuges as before. Resulting supernatant is Triton-solubilized material. An aliquot of Triton solubilized material was digested with trypsin for 1 h at 37°C followed by a 5-fold excess of soybean trypsin inhibitor. All samples were assayed by radioimmunoassay (RIA) in duplicate at four different sample volumes. Nonspecific binding was <3% of total. Duplicates agreed within 5%, and separate volumes agreed within 15%. Values presented are means ± SE; $n = 15$ for both groups. Open bars, sham-operated control animals; solid bars, postischemic rats.

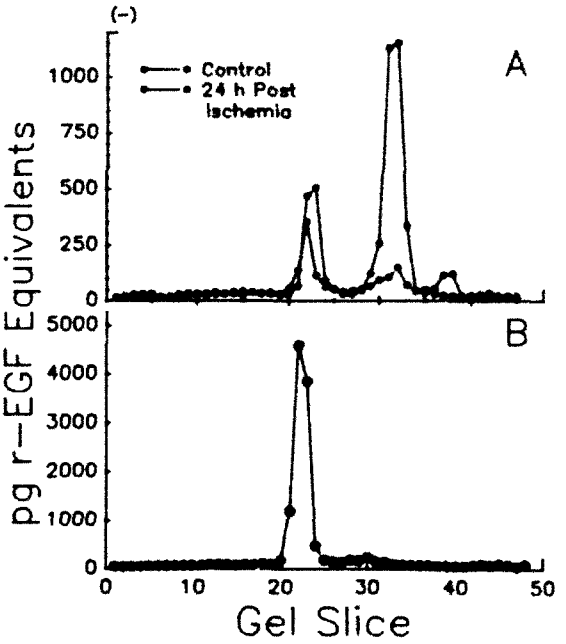

Fig. 2. Native gel electrophoresis of affinity purified immunoreactive (ir) EGF. Initial cytosolic supernatant was affinity purified with antisera directed against submandibular gland EGF. Immunoreactive material was electrophoresed as in MATERIALS AND METHODS. Each lane was cut into 3-mm slices and extracted in 1.0 ml RIA buffer at 4°C and assayed in duplicate by RIA. Submandibular gland migrates at *slice 22*, rat des-Arg-EGF migrates at *slice 32*, and dye front was at *slice 42*. *A*: irEGF from *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid extract after centrifugation. *B*: electrophoretic profile of irEGF from affinity-purified Triton X-100 extract that was digested with trypsin before affinity extraction.

EGF, with a lesser amount of material comigrating with des-Arg-EGF. In contrast, the injured kidney contains a majority of immunoreactive material comigrating with the des-Arg form, with a lesser amount comigrating with

native EGF. A third form, that migrated at *slice 38* was present in varying amounts (20% of total activity in 1 run, but usually <5%) in the injured kidneys and was not further characterized. HPLC molecular mass sieve chromatography was conducted on the affinity-purified soluble EGF from these same preparations. The irEGF from the control and injured kidneys contained material that coeluted with native EGF (data not shown). This method is not capable of separating EGF from des-Arg-EGF because of their similar sizes.

The major soluble immunoreactive forms from the postischemic kidneys were electroeluted from nondenaturing gels as described in MATERIALS AND METHODS and assayed by RIA (Fig. 3). Both major peaks demonstrated parallel displacement with submandibular gland EGF, supporting their identification as EGF and des-Arg-EGF. Des-Arg-EGF demonstrates parallel displacement in the RIA (data not shown). A portion of the lane containing no activity did not interfere with the assay. Biological activity of the electroeluted fractions was assayed by adding aliquots to growth-arrested human fibroblast cell line JPS1 and measuring the incorporation of [^{3}H]thymidine (Table 1). In control samples, stimulation by EGF was detected at 0.1 ng/ml, with a maximum of a sevenfold increase in [^{3}H]thymidine incorporation at 10 ng/ml. The electroeluted material from a region of the gel containing no immunoreactive material had no effect on the level of thymidine incorporation. Both electroeluted fractions of immunoreactive material were biologically active and stimulated the incorporation of thymidine in a dose-dependent manner (Table 1).

Quantitation of irEGF in Triton-solubilized membrane. The amount of irEGF in Triton-solubilized crude membrane pellets indicated a slight, but not statistically significant, decrease in the injured animals (23.9 ± 3.0 vs. 18.8 ± 3.0 ng irEGF eq/g wet wt; Fig. 1). The displacement curves generated by the Triton-solubilized membranes were not parallel to the standard curve, indicating nonidentity with submandibular gland EGF. The nonparallel displacement is expected and is most probably due to the tertiary structure of the precursors, which prevents the antibodies from reaching all of the antigenic

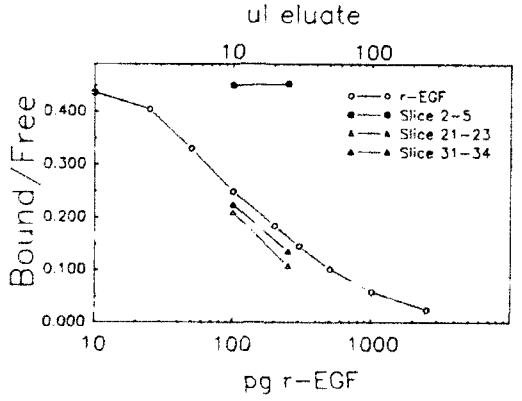

Fig. 3. RIA of affinity-purified native gel-separated cytosolic irEGF. Affinity-purified cytosolic irEGF was separated by native gel chromatography as in Fig. 2. Activity was electroeluted and assayed by RIA to demonstrate parallel displacement. A section of lane that had no activity serves as a negative control (*slices 2–5*).

Table 1. *Stimulation of [^{3}H]thymidine incorporation into growth-arrested fibroblasts*

Addition	dpm × 10^3
Control	4.08 ± 0.24
Rat EGF	
0.1 ng/ml	14.44 ± 0.60
1.0 ng/ml	26.49 ± 2.45
10 ng/ml	32.71 ± 0.91
FBS (10%)	26.71 ± 1.02
Slices 2–5	
50 μl	4.91 ± 0.72
Slices 22–25	
5 μl	10.11 ± 1.79
50 μl	25.82 ± 2.46
Slices 32–35	
5 μl	4.89 ± 0.12
50 μl	36.94 ± 0.57

Values are averages of 3 measurements ± SEM. Growth arrested JPS1 fibroblasts were incubated with the indicated amounts of rat epidermal growth factor r (EGF), fetal bovine serum (FBS), or electroeluted material from native gel. *Slices 2–5* contained no immunoreactive material and served as a negative control. *Slices 22–25* represent migration rate of standard rat EGF; *slices 32–35* represent migration rate of des-Arg standard rat EGF. Trichloroacetic acid-insoluble NaOH soluble material was extracted as in MATERIALS AND METHODS.

determinants of the mature EGF molecule resulting in the inability to fully compete with ^{125}I-rat EGF in the RIA.

We previously demonstrated multiple forms of biologically active irEGF in milk, which also demonstrated nonparallel displacement by RIA before trypsin digestion. These multiple forms were converted to the native form by digestion with trypsin (29). We have also determined that submandibular gland native EGF is resistant to trypsin digestion (31). In contrast, the mouse EGF precursor can be converted to a 6-kDa form by pepsin digestion (3). Triton X-100 extracts from control and postischemic samples were digested with trypsin to determine whether the kidneys contained EGF precursors. When the detergent-solubilized material from control samples was digested with trypsin, the level of immunoreactive material increased 13-fold to 301 ± 31 ng/g wet wt. When Triton-soluble material from postischemic kidneys was trypsinized, there was a fourfold increase to 75.7 ± 19.5 ng/g wet wt (Fig. 1). This indicates a >75% loss of membrane-associated EGF in the injured kidneys.

Characterization of trypsinized Triton-solubilized irEGF. To determine the nature of the immunoreactive form(s) generated by trypsin digestion of the Triton X-100 extract, and to test the possibility of generation of multiple immunoreactive peptides, the Triton X-100 extract from the control kidney was first digested with trypsin and subsequently affinity purified and electrophoresed. Only one appreciable peak of immunoreactive material that comigrated with rat submandibular gland EGF was present, demonstrating that the only detectable immunoreactive form in the trypsinized Triton X-100 extract has an identical charge to mass ratio as native rat EGF (Fig. 2*B*). A similar profile was obtained using samples from the ischemia-injured kidneys (data not shown).

This method cannot rule out the possibility of the presence of small number of uncharged amino acids at the NH_2-terminal of the polypeptide, which would not alter the migration rate of the molecule.

When the affinity-purified Triton X-100 extract was subjected to electrophoresis before trypsin digestion, there was a smearing of activity on the gel with few distinguishable peaks of activity (data not shown).

HPLC molecular sieve chromatography of Triton-solubilized irEGF. Because the membrane-bound putative EGF precursor is not highly immunoreactive (Fig. 1), we used a large excess of affinity resin to remove all EGF-containing forms in the Triton-solubilized crude membrane preparation. This affinity-purified Triton-solubilized membrane preparation was characterized by HPLC sizing chromatography using a Bio-Sil 250 column. Aliquots of each eluate fraction were removed for RIA and also for trypsin digestion with subsequent assay by RIA. It is important to note that trypsin digestion of the fractions was performed after the material had eluted from the column on the basis of their respective molecular masses. The results from the control kidney are illustrated in Fig. 4*A*. Direct assay of the column eluate indicated a major peak of activity eluting at 16 min with a lesser amount eluting at 22 min. When the same eluate fractions were digested with trypsin before RIA, the activity profile was strikingly different. The immunoreactive material eluting at 11.5 min increased from 50 ng/fraction to >1,000 ng in the same fraction. The 16-min peak increased in intensity approximately threefold. The material eluting at 22 min was unaffected by trypsin digestion (this is the retention time for rat EGF). A fourth peak of activity was eluted at 39 min. This material eluted at 1.8 column volumes and was consistent between runs. The retention time was not affected by longer wash periods between runs. This peak is apparently larger than that for rat EGF because of the activation by trypsin and is therefore being retained by the column by factors unrelated to molecular mass.

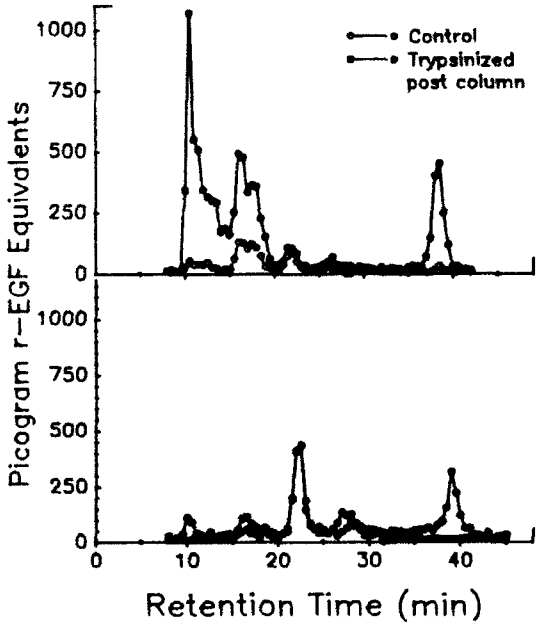

Fig. 4. High-performance liquid chromatography (HPLC) of affinity-purified Triton-solubilized membranes. Affinity-purified material was chromatographed by HPLC fractionation as in MATERIALS AND METHODS. Aliquots of individual fractions eluting from column were either assayed directly by RIA or digested with trypsin to generate low-molecular-mass EGF and subsequently assayed by RIA. *A*: normal kidney; *B*: 24 h postischemia. Pools were made of material eluting between 10.5 and 14 min (*pool 1*), 15.5 and 19.0 min (*pool 2*), 21.0 and 23.5 (*pool 3*), and 37.0 and 39.5 (*pool 4*). Four pools of activity were used for additional characterization.

When the affinity-purified Triton X-100 material from postischemic kidneys was characterized by HPLC fractionation as above, the pretrypsin activity profile was not substantially different from the corresponding control profile (Fig. 4*B*). This is consistent with the RIA results for the pretrypsinized Triton X-100 extract. However, in contrast to the control kidney, there were only slight increases in the amount of immunoreactive material generated by trypsin digestion. Again, it is important to note that the trypsin digestion was performed on the fractions after they had been separated by the column based on their apparent molecular masses. The native EGF peak at 22 min is larger than in control kidneys and remains unchanged, and the peak at 39 min demonstrates an increase in activity. Some postischemic kidneys had a slightly greater amount of the 16-min fraction but all were >80% decreased in the 9.5-min peak.

The Bio-Sil 250 column was calibrated with proteins of known masses. The results were as follows: thyroglobulin, 669 kDa, retention time 10.8 min; ferritin, 440 kDa, retention time 13.0 min; catalase, 232 kDa, retention time 15.6 min; aldolase, 158 kDa, retention time 16.3 min; rat EGF, 6 kDa, retention time 22.5 min; and phenylalanine 165 Da, retention time 25.6 min. We do not know whether the putative precursors share this behavior. Therefore apparent molecular masses for the precursors have been assigned, based on the standards listed above: *peak 1*, 500 kDa; *peak 2*, 150 kDa; *peak 3*, 6 kDa; *peak 4* is unknown but must be >6 kDa because it is converted to 6 kDa by trypsin. The apparent molecular mass of *peak 2* is consistent with literature values of 140-150 kDa for the rat EGF precursor found in kidney and urine (17). The retention times were extremely consistent between runs but the molecular mass values are termed "apparent" because of EGF's known anomalous behavior on column chromatography (26, 30, 31).

Characterization of Triton-solubilized irEGF from control kidney membrane preparations. To generate sufficient material for biochemical analyses, Triton X-100 extracts from a homogenate of 12 kidneys were affinity purified and chromatographed in two successive HPLC runs. Pools were made of material eluting between 10.5 and 14 min (*pool 1*), 15.5 and 19.0 min (*pool 2*), 21.0 and 23.5 (*pool 3*), and 37.0 and 39.5 (*pool 4*). Each pool of activity was lyophilized and resuspended in 1.0 ml Eagle's MEM containing 0.2% bovine serum albumin, and an aliquot was removed for trypsin digestion. The trypsin-treated and untreated pools were used for further analysis to determine relative activities of the fractions.

Radioimmunoassay. *Pools 1–4*, both trypsinized and undigested, were diluted 1:10 and assayed by RIA. The results are illustrated in Fig. 5. *Pool 1* contained 0.14 ng/ml rat EGF eq. This activity was increased 80-fold by trypsin digestion to 11.14 ng/ml. *Pool 2* contained 2.03 ng/ml, which increased to 7.06 ng/ml after trypsin digestion. *Pool 3* demonstrated only a moderate increase after trypsin digestion, from 1.05 to 1.45 ng/ml. This peak is presumed to represent native EGF. The slight increase may be due to cross-contamination from *peak 2*. *Pool 4* increased 27-fold (from 0.07 to 1.94 ng/ml) after trypsin digestion. All four of the trypsinized fractions demonstrated parallel displacement with rat submandibular gland EGF after digestion. *Pools 2* and *3* demonstrated parallel displacement before trypsin digestion. These results indicate that the 150-kDa form and native EGF are structurally similar regarding exposure of immunoreactive determinants. The observation that the 500-kDa form does not generate parallel displacement along with the 80-fold increase in activity after trypsinization supports the hypothesis that this form is not an aggregate of the 150-kDa form.

Radioreceptor assay. The pools of activity were tested for their ability to compete with ^{125}I-rat EGF for binding to the EGF receptor on cultured human fibroblasts (Fig. 6). *Pool 1* had minimal binding activity using either a 10- or 50-μl aliquot of material. Digestion of *pool 1* with trypsin resulted in activity equal to 100 ng/ml rat EGF, representing >20-fold increase in activity. Displacement of ^{125}I-rat EGF by *pool 2* indicated it contained 10 ng EGF/ml. Trypsin digestion increased this activity fivefold to 50 ng/ml. *Pool 3* contained ~100 ng/ml rat EGF and was unchanged by trypsin digestion. *Pool 4* had no measurable activity before digestion and increased to 25 ng/ml after trypsinization. These results indicate that the two high-molecular-mass EGF-containing fractions are able to compete with radiolabeled rat EGF for the EGF receptor and that their ability to compete is substantially enhanced after limited proteolysis. These results are consistent with the RIA results that demonstrated increased activity after trypsin digestion.

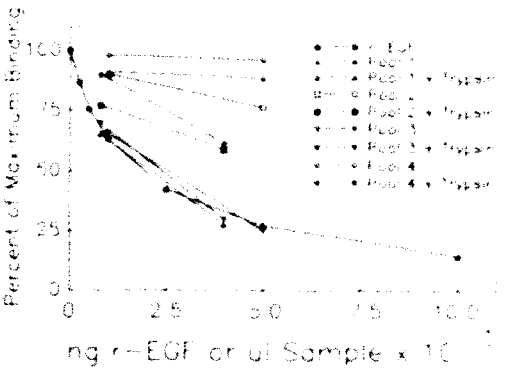

Fig. 6. Radioreceptor assay of HPLC-fractionated immunoreactive material pools of immunoreactive material were generated as outlined in MATERIALS AND METHODS. Aliquots of either rEGF or pools of activity were assayed for their ability to compete with ^{125}I-rEGF for binding to cultured fibroblasts as described in MATERIALS AND METHODS. Data points represent average of duplicate determinations. Nonspecific binding was determined in presence of a 1,000-fold excess of rEGF and was <2% of total binding.

[^{3}H]thymidine incorporation. Growth-arrested human foreskin fibroblast cell line JPS1 was exposed to the Triton-extracted EGF fractions to test their ability to stimulate DNA synthesis. The results of this assay are illustrated in Fig. 7. Both the 500- and 150-kDa precursors were capable of stimulating [^{3}H]thymidine incorporation in a dose-dependent manner. The activity of the 500-kDa precursor was increased from a 40% stimulation by a 10-μl aliquot of *pool 1* to a sevenfold increase after the sample was digested with trypsin. The increase in activity after trypsin treatment of the 150-kDa precursor was only evident at the lower concentration. The activity of the fraction comigrating with native EGF was unaffected by trypsin. The material in *pool 4* demonstrated a slight dose-dependent increase in stimulation. The activity in *pool 4* was significantly enhanced after proteolysis.

Comparisons with known high-molecular-mass renal proteins. We tested the possibility that the high-molecular-mass EGF-containing protein may represent a previously characterized renal membrane protein. One high-molecular-mass protein present in both kidney and urine

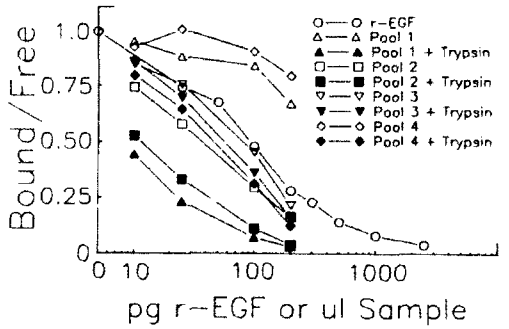

Fig. 5. RIA of HPLC-fractionated immunoreactive material from control rat kidneys. Pools of immunoreactive material were generated and digested with trypsin as in MATERIALS AND METHODS. Material eluting from HPLC fractionation indicated in Fig. 4 eluting between 10.5 and 14 min represents *pool 1*, 15.5 and 19.0 min represents *pool 2*, 21.0 and 23.5 represents *pool 3*, and 37.0 and 39.5 represents *pool 4*. Aliquots of each of the pools were tested for their ability to compete with ^{125}I-rEGF for binding antibodies generated against native rEGF. Data points represent average of duplicate determinations.

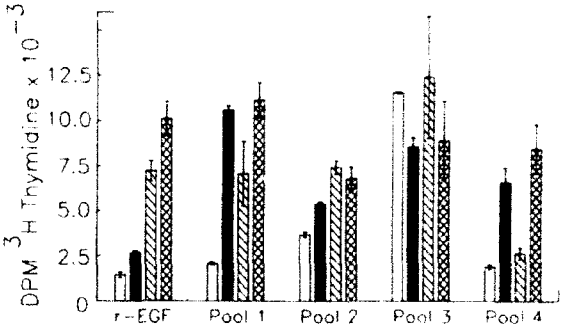

Fig. 7. Stimulation of [^{3}H]thymidine incorporation into growth-arrested fibroblasts by HPLC-fractionated immunoreactive material. Pools of immunoreactive material were generated as in MATERIALS AND METHODS. Stimulation of [^{3}H]thymidine incorporation was conducted as in MATERIALS AND METHODS. Additions were as follows: standard rEGF, open bar no addition; solid bar 0.1 ng/ml; hatched bar 1.0 ng/ml; cross-hatched bar 10.0 ng/ml. Aliquots of 4 pools were as follows: open bar 10 μl of pool; solid bar 10 μl trypsin-digested pool; hatched bar 50 μl of pool; cross-hatched bar 50 μl trypsin-digested pool.

is the Tamm-Horsfall protein, (for a recent review see Ref. 16). This polymeric protein has been reported to contain cysteine-rich regions which are homologous to the EGF precursor (34). A sample of the rat Tamm-Horsfall protein was generously provided by Dr. John Hoyer, Children's Hospital, University of Pennsylvania. Neither the Tamm-Horsfall protein nor the trypsinized product competed with labeled rat EGF in the RIA when used at concentrations 1,000-fold the level of sensitivity of the assay. Rat kidney membranes and urine contain a high-molecular-mass protein termed gp330 whose function is unknown (11). A sample of rat gp330 was generously provided by Dr. Robert McCluskey, Harvard Medical School. Neither the intact gp330 nor the trypsin-generated fragments competed in the rat EGF RIA at concentrations 10,000-fold greater than the limit of detection on a per-weight basis. In addition, polyclonal antisera to gp330 provided by Dr. John Niles, Massachusetts General Hospital, did not bind radiolabeled rat EGF.

DISCUSSION

These results demonstrate a sixfold increase in biologically active soluble EGF in the rat kidney 24 h postischemic injury. This soluble EGF is present in two distinct forms. It is not known whether the des-Arg form of EGF is generated as such or whether it is generated from the intact native form. The des-Arg form of EGF is generated in the endosomal compartment of fibroblasts shortly after internalization (28). Local action of EGF would be consistent with the reported increase in EGF binding capacity of renal membranes after ischemic injury (23) as well as folic acid-induced injury (1) and may partially explain a rise in renal EGF during a time when urinary levels of EGF decrease (24). The decrease in urinary EGF suggests the presence of a mechanism by which EGF is either not released into the lumen or is sequestered back into the tissue by a currently unknown mechanism.

The increase in soluble irEGF reported here occurs during a time period when mRNA for the EGF precursor has been reported to be <1% of the control levels (23). These apparently disparate results prompted us to determine the source of the renal EGF. Serum levels of EGF are <1.0 ng/ml (27) and thus are an unlikely source for the observed renal EGF levels. Initial examination of the Triton-solubilized crude membrane preparations indicated only a slight decrease in immunoreactive material in the injured kidneys. However, when these same preparations were digested with trypsin there was a substantial difference between the control and ischemia-injured kidney. Digestion of the solubilized membrane fraction from either the control or injured kidney generates a single immunoreactive form which is identical to rat submandibular gland EGF by RIA, radioreceptor assay, and electrophoretic migration in a native, nondenaturing polyacrylamide gel. The level of EGF in the control kidney membranes, >300 ng/g, represents >90% of total renal EGF and is sufficient to account for the increase seen in the injured kidney. Total EGF in the injured animal (soluble + trypsin generated EGF) is less in the injured animals than the controls. These data are consistent with EGF binding its receptor and being degraded in the lysosome resulting in an overall decrease in irEGF.

HPLC analysis of the control Triton X-100 irEGF identified an EGF-containing protein with an apparent mass of 150 kDa as described in the mouse (3), as a previously uncharacterized form with an apparent mass of 500 kDa. The 500-kDa form does not appear to be an aggregate of the 150 kDa for the following reasons: 1) the 500-kDa form does not demonstrate parallel displacement in the RIA, whereas the 150-kDa form does; 2) the magnitude of the increase after trypsinization as measured by RIA, radioreceptor assay, and thymidine incorporation are substantially different between the two forms; 3) rechromatography of the 500-kDa peak of material results in a single peak of activity with the same retention time (data not shown). As previously mentioned, it is possible that EGF is complexed with a separate protein by interactions which are stable to heating in 1% SDS. It is also important to note that EGF is well known to demonstrate anomalous behavior on column chromatography (26, 30, 31).

A 500-kDa form has not been reported in mice or humans. This form may be unique to rats or it may be that, because of its large size and lower level of immunoreactive abundance relative to the 150-kDa form, it has been undetected. This represents a novel form of EGF that contains a substantial portion of EGF within the rat kidney membrane. The large size of the 500-kDa precursor combined with the substantial amount of EGF liberated after limited proteolysis suggests that this molecule may have multiple EGF repeats which would distinguish it from the 150-kDa precursor. Both precursors have biological activity as determined by the ability to stimulate [^{3}H]-thymidine incorporation into growth-arrested human fibroblasts. Direct comparisons of activities cannot be made because of the harsh treatment required to remove the activity from the affinity resin at neutral pH. However, the levels of irEGF in the Triton X-100 extract were indistinguishable from that displaced from the resin indicating that there was no net change in the number of antigenic determinants presented on the surface of the molecules.

The role of proteolytic enzymes in the release of biologically active peptides has been recently reviewed (8). This combined with the known existence of numerous membrane-anchored proteins that contain multiple EGF-like repeats, reviewed by Massague (18), suggests a potential role for proteases in releasing high concentrations of biologically active molecules from latent precursor stores. This type of mechanism would allow for the release of substantial amounts of growth promoting agents with a minimal energy requirement of the cell. Membrane-anchored precursors for both human EGF and transforming growth factor-α (TGF-α) have been demonstrated to have inherent biological activity (2, 20). The TGF-α precursor has been proposed as a model for membrane-anchored growth factors (18), and its activities are dependent on the site and extent of proteolytic cleavage. The recent report of a membrane-associated enzyme that releases biologically active EGF from kidney membranes (13) as well as the observation that aprotinin, a trypsin inhibitor, decreases the urinary level of rat EGF (14) support the existence of

the protease required for the release of EGF from kidney membranes.

These results also offer an explanation for the decrease in renal prepro-EGF mRNA noted at a time when other transcriptional activity is increased (23). The authors suggested that this may be due to a negative effect of the regenerative process on transcription of this gene or due to the interruption of EGF processing. Our observations suggest that decreased transcription could be due to simple end-product inhibition resulting from the release of biologically active EGF from its membrane-bound precursors.

Recently hepatocyte growth factor (HGF) was proposed to be a mediator of renal hypertrophy after unilateral nephrectomy (21). The levels of mRNA for HGF as well as the active growth factor increase within hours after renal injury. The time frame of HGF appearance supports its role as a growth-stimulating factor in recovery from acute renal failure. EGF also appears to be a likely candidate for the increases in DNA synthesis 48 h after renal injury. It is also possible that the two growth factors work in concert to stimulate the repair of the injured tissue.

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